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ABSTRACT

The WNT family activates an oncogenic signaling mediated through β -catenin and is up-regulated in a variety of malignant neoplasms. The signaling translocates β -catenin into the nucleus and stimulates carcinoma cells in the epithelial-mesenchymal transition (EMT). However, WNT expression and signaling in oral carcinomas have not been examined. The present study focused on unveiling the involvement of WNTs in oral carcinomas, and showed that carcinoma cells express 11 of 19 WNT family members by reverse-transcription/PCR. WNT-expressing carcinoma cells exhibited increased β -catenin levels in the cytoplasmic pool and translocation to the nucleus. The activation state of signaling correlated with the expression of membrane-type 1 matrix metalloproteinase, which degrades territorial matrices in carcinoma invasion. Immunohistochemistry disclosed that WNT3 expression and nuclear localization of β -catenin were predominant in carcinoma cells at the invasive front. These results suggest that enhanced WNT expression and signaling accelerate the progression of carcinomas *via* activating EMTs and local invasiveness.

KEY WORDS: β -catenin, EMT, invasion, oral carcinoma, WNT.

Activation of WNT Family Expression and Signaling in Squamous Cell Carcinomas of the Oral Cavity

INTRODUCTION

Oral squamous cell carcinoma is the most common neoplasm of the head and neck. Carcinoma cells accumulate a series of genetic and/or epigenetic changes and altered phenotypes during tumor progression. Loss of the epithelial morphology and acquisition of mesenchymal characteristics, epithelial-mesenchymal transition (EMT), are typical for carcinoma cells during tumor progression and correlate with local invasiveness and metastatic potential of the tumor. Such changes will associate with aberrant expression of developmentally regulated and mesenchymal cell-related genes (Birchmeier *et al.*, 1996; Mizunuma *et al.*, 2003). Studying the gene expression involved in EMTs may provide insights into the mechanism of tumor progression.

The WNT family has a crucial role in development and constitutes 19 members in the human genome. WNT1 was identified as an oncogenic gene activated by chromosomal integration of Mouse Mammary Tumor Virus. Ligation of WNT to cell-surface receptors, FRIZZLED (FZ) and LDL receptor-related protein, sparks signaling pathways mediated by β -catenin (CTNNB1) or others (Seidensticker and Behrens, 2000). CTNNB1-mediated signaling has been characterized extensively (Seidensticker and Behrens, 2000). In this pathway, FZ abrogates kinase activity of glycogen synthase kinase 3- β (GSK3 β), liberating CTNNB1 from degradation and increasing the cytoplasmic-free CTNNB1 pool. An excess amount of CTNNB1 translocates into the nucleus and transcribes target genes. In the absence of WNT, GSK3 β forces CTNNB1 to degrade, resulting in a decrease of the free CTNNB1 pool (Seidensticker and Behrens, 2000; Lo Muzio, 2001).

WNT signaling initiates proliferation, dedifferentiation, and EMTs in various types of carcinoma cells (Eger *et al.*, 2000; Lo Muzio, 2001). Recent evidence increases a panel of WNT target genes, such as c-MYC and extracellular matrix (ECM)-degrading endoproteases (www.stanford.edu/~russel/wntwindow.html). c-MYC is involved in tumorigenesis and cell proliferation (Lutz *et al.*, 2002). Degradation of the ECM is an indispensable step in tumor invasion and metastasis, and matrix metalloproteinases (MMPs) have a central role in such degradation (Imai *et al.*, 1995a, 1997a; Ohuchi *et al.*, 1997). Recent studies emphasize a role for membrane-type 1 MMP (MT1-MMP) in cancer cell invasion and progression of other diseases (Imai *et al.*, 1996, 1997b; Ueno *et al.*, 1997). It is known that WNT signaling directly up-regulates MT1-MMP expression (Takahashi *et al.*, 2002). Therefore, activation of the WNT signaling pathway can have a significant impact on tumor progression. However, no examination has been made of WNT expression in oral carcinomas (Lo Muzio, 2001). In the present study, we developed an expression panel of WNT family members and demonstrated activation of WNT signaling in carcinoma cells. Immunohistochemical staining indicated that the signaling was prominent at the invasive front in the carcinoma tissues.

Table. Primer Sequence for WNT Genes and Predicted Size of PCR Amplicons

Gene	Primer Sequence	Product Size (bp)
WNT1	Forward 5'-TCCTGCTCAGAAGGTTCCAT	483
	Reverse 5'-GCTGTACCTGCGAGAGTTGG	
WNT2	Forward 5'-CTGTATCAGGACCGAGAGG	500
	Reverse 5'-CAAAGAGAAGCTGCCAGGAG	
WNT2b	Forward 5'-ACTGAGTGTGTGCGAGTGTG	502
	Reverse 5'-TGATGTCTTGTGACAGACAC	
WNT3	Forward 5'-ACTTCGCGGTGTTAGTGTCC	501
	Reverse 5'-ATTITTCCTTCCTGCTTCCTC	
WNT3a	Forward 5'-TGCACCTCATCCAGCTACAG	497
	Reverse 5'-GAATTTGAGGCGAGGATGG	
WNT4	Forward 5'-TTGAGGAGTGCCAGTACACG	565
	Reverse 5'-TTGACATGTGCGTGTGCGTGG	
WNT5a	Forward 5'-CAGTTCAAGACCGTCGACAG	501
	Reverse 5'-TGGAACTACCACTCCCATTA	
WNT5b	Forward 5'-GTGCTGCTTCGTCAGGTGA	497
	Reverse 5'-CGAGGTGAAGCTGAGTTC	
WNT6	Forward 5'-CAACTGCACAACACGAGGC	445
	Reverse 5'-GTACTACGCGACCGAGTGG	
WNT7a	Forward 5'-GAGAAGCAAGGCCATGACCA	522
	Reverse 5'-ACAGCACATGAGGTGACAGC	
WNT7b	Forward 5'-TGCCAGCAGCAGACATAGAC	504
	Reverse 5'-AGAGGGTGTCTAGGGGATGG	
WNT8a	Forward 5'-AGATGCTATCAGCTCTGCTG	490
	Reverse 5'-AAAGATCAGTTCGCTCTCTG	
WNT8b	Forward 5'-GACACCTTCGCTCCATCTC	503
	Reverse 5'-GAAAGTGGCAAGCTTTGGAG	
WNT10a	Forward 5'-AATGAGGCTTCACAACCAAC	654
	Reverse 5'-TCATGTGGTCCAATCTCTCT	
WNT10b	Forward 5'-CTTCATTGATACCCACAACC	518
	Reverse 5'-ATTGTGGGAGAGAGGCTAC	
WNT11	Forward 5'-TGACCTCAAGACCCGATACC	510
	Reverse 5'-CAAGTGGAAGGCAAGACACAA	
WNT14	Forward 5'-AAGATGTGCGCACTTACCC	507
	Reverse 5'-TAAGGAACCCAGCCAGACAC	
WNT15	Forward 5'-TGCACTGTGATGACTCTCC	491
	Reverse 5'-GAGCACACCTTACCTGCTGT	
WNT16	Forward 5'-GTGACACCACTTGTGACAGAC	503
	Reverse 5'-ACCTCTGTGATGACGTTGCG	
GAPDH	Forward 5'-GTCAGTGGTGGACAGCACT	395
	Reverse 5'-AGGGGAGATTGAGTGGTGG	

MATERIALS & METHODS

Cell Lines and Tissue Samples

Oral squamous carcinoma cell lines (Ca9.22, Ho1u1, HOC313, HSC2, HSC3, KOSC2, KOSC3, OSC19, SCCKN, SCC1F, and TSU) were obtained from the Cell Resource Center (Sendai, Japan), the Health Science Research Resources Bank (Osaka, Japan), or the RIKEN Cell Bank (Tsukuba, Japan). Normal gingival fibroblasts (GF12) (Takahashi *et al.*, 1997) were used as a control. Normal gingival keratinocytes from patients undergoing dental surgery at Nippon Dental University Hospital were obtained under an informed consent protocol that was reviewed and approved by the Institutional Review Board, and were primary-cultured in defined keratinocyte-SFM (Invitrogen, Grand Island, NY, USA).

Reverse-transcription/PCR

Total RNA was isolated with the use of TRIzol reagent (Invitrogen) followed by RNase-free DNase I treatment. After reverse transcription (RT) with SuperScript II and random hexamer (Invitrogen), we performed PCR amplification by running 30 cycles under the following conditions: 94°C for 40 sec, 54°C for 40 sec, and 72°C for 1 min. Gene-specific primer sets for WNTs or GAPDH are listed in the Table.

Tissue Specimen Section and Immunohistochemistry

Incisional or excisional biopsy specimens from 42 patients with oral squamous cell carcinomas were collected from the files of Kanazawa University Hospital. Clinical and pathological data were obtained from the patients' medical records and The Kanazawa University Hospital Surgical Pathology Files. These samples were immediately fixed in 10% neutral buffered-formalin and embedded in paraffin wax. Unstained tissue sections (4 µm) were incubated with goat anti-WNT3 (10 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-CTNNB1 (5 µg/mL, BD Bioscience, San Diego, CA, USA) antibodies for 12 hrs at 4°C. Biotinylated anti-goat or -mouse IgG antibodies (DAKO, Glostrup, Denmark) were used for secondary antibody, followed by incubation with avidin-biotin complexes (Vector Laboratories, Burlingame, CA, USA). The color was developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA) and counterstained with hematoxylin.

Immunocytochemistry

Carcinoma cell lines (TSU, HOC313, HSC2, and SCCKN) were cultured on glass slides (Lab-Tek Chamber II, NUNC, Naperville, IL, USA) and fixed with 1% paraformaldehyde. The cells were reacted with primary antibodies to WNT3 (10 µg/mL), CTNNB1 (4 µg/mL), or GSK3B (4 µg/mL, Cell Signaling Technology, Beverly, MA, USA) for 16 hrs at 4°C. Alexa Fluor 546 anti-rabbit or -goat IgG or Alexa Fluor 488 anti-mouse IgG antibodies (Molecular Probes, Eugene, OR, USA) were used for secondary antibody.

Immunoblotting

Nuclear extracts of TSU, HOC313, HSC2, or SCCKN cells were prepared by the method described elsewhere (Lee *et al.*, 1988). Total cell lysates (35 µg) or nuclear extracts (20 µg) were size-fractionated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. The membrane was probed with anti-CTNNB1, -GSK3B, or -β-actin (Sigma-Aldrich) antibodies and then with biotinylated secondary antibodies, and the color developed with avidin-biotin-complex and 3,3'-diaminobenzidine tetrahydrochloride.

RESULTS

Expression of the WNT Family in Oral Carcinomas

We performed RT-PCR for WNT family members and generated a panel of the expression pattern (Fig. 1A). Among 19 members of the family, WNT3, 3a, 4, 5a, 5b, 6, 7a, 7b, 10b, 11, and 14 were amplified in all of the carcinoma cell lines, except for HSC2 cells. Normal keratinocytes expressed

WNT5a, 5b, 6, and 7a, and GF12 gingival fibroblasts amplified WNT2, 2b, 3, 5a, 5b, 11, 14, and 16 (Fig. 1B). Keratinocyte-type WNTs (6 and 7a) were most commonly expressed, and fibroblast-type WNTs (3, 11, and 14) were less frequently expressed. Other cell-type WNTs (3a, 4, 7b, and 10b) were also ectopically identified in the carcinoma cell lines examined. Specific amplification of each WNT gene was clarified by sequence analysis (data not shown).

Activation of WNT Signaling Pathway

We examined protein expression by immunohistochemistry and Western blot WNT3-specific antibody stained HOC313 and TSU cells, but not SCCKN and HSC2 cells (Fig. 2A). Immunostaining and Western blot analyses showed weak reduction of GSK3B in HSC2 cells (Figs. 2A, 2B). However, immunostaining of CTNNB1 in WNT-expressing HOC313, TSU, and SCCKN cells exhibited accumulation in the nucleus and diffuse cytoplasmic distribution (Fig. 2A). In contrast, HSC2 cells localized CTNNB1 at the cell-cell boundaries. Since CTNNB1 is crucial for the formation of E-cadherin-mediated cell-cell adhesion (Nagafuchi *et al.*, 1994), it is bifunctional, undertaking WNT signaling and cell-cell adhesion (Gottardi *et al.*, 2001). Western blot analysis also showed that increased amounts of the free CTNNB1 pool were in the WNT-expressing cells compared with HSC2 cells (Fig. 2B). Nuclear extracts of HOC313, TSU, or SCCKN cells reacted to CTNNB1 antibody, whereas no reaction was observed in the HSC2 cells.

Activation of Downstream Targets of WNT Signaling

Analysis of our data above demonstrates the activation of the WNT signaling pathway in oral carcinoma cells. Among WNT target genes, c-MYC oncogene and MMPs have been well-documented to accelerate tumor progression (Ueno *et al.*, 1997; Shimada *et al.*, 2000; Lutz *et al.*, 2002). Therefore, we examined the expression of c-MYC and MT1-MMP in the cell lines expressing WNT genes. Accumulation of c-MYC in the nucleus was evident in TSU and HOC313 cells but not in HSC2 cells. Unexpectedly, c-MYC was negligible in SCCKN

Figure 2. Activation of the WNT signaling and downstream target genes.

(A) Immunocytochemistry of WNT3 (a,d), GSK3B (b,e), and CTNNB1 (c,f) in carcinoma cells. WNT-negative HSC2 cells (a-c) and WNT-expressing TSU cells (d-f) cultured on glass slides were applied for fluorescent immunostaining. Bar = 25 μ m. (B) Analysis of WNT signaling activation in carcinoma cells. Total cellular lysates or nuclear extracts from HOC313 (lane 1), TSU (lane 2), HSC2 (lane 3), or SCCKN (lane 4) were subjected to Western blot. (C) Expression of WNT target genes in carcinoma cells. Size-fractionated protein of HOC313 (lane 1), TSU (lane 2), HSC2 (lane 3), or SCCKN (lane 4) was reacted to specific antibody to c-MYC or MT1-MMP. c-MYC was localized to the nuclei of TSU cells (b), but not to HSC2 cells (a). Bar = 25 μ m.

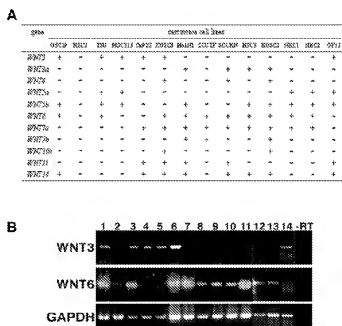
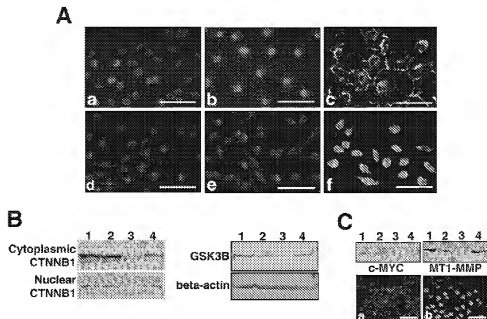


Figure 1. Expression of WNT family members in oral squamous carcinoma cell lines. (A) A panel of WNT members amplified by RT-PCR is listed in the Table. Normal keratinocytes (NK1 and NK2) and fibroblasts (GF12) isolated from gingiva were included. (B) Representative data of RT-PCR for WNT3, WNT6, and GAPDH as an internal control (lane 1, OSC19; lane 2, HSC2; lane 3, TSU; lane 4, HOC313; lane 5, Ca9.22; lane 6, KOSC3; lane 7, Hs1u1; lane 8, SCC1F; lane 9, SCCKN; lane 10, HSC3; lane 11, KOSC2). RNA sample isolated from normal gingival keratinocytes (lane 12, NK1; lane 13, NK2) and GF12 normal gingival fibroblasts (lane 14) were also applied. PCR reactions without reverse transcription of OSC19 cells were also performed as a negative control [-RT].

cells (Fig. 2C). Western blotting analyses also showed selective expression of MT1-MMP in the WNT-expressing TSU, HOC313, and SCCKN cells (Fig. 2C).

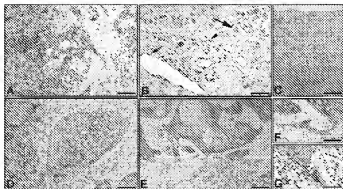


Figure 3. Immunolocalization of WNT3 or CTNNB1 in oral carcinoma tissue. (A) WNT3 was localized to carcinoma cells at the invasive front. (B) Endothelial cells (small arrow), fibroblast-like cells (arrowhead), and macrophage-like cells (large arrow) adjacent to carcinoma cells were also positively stained. (C) Normal gingiva did not react to WNT3 antibody. (D,E) CTNNB1 stained cell-cell junction of carcinoma cells. (F,G) At the extremity of carcinoma invasion, CTNNB1 showed diffuse cytoplasmic or nuclear staining. Bar = 100 μ m (A,C-E) and 50 μ m (B,F,G).

Immunostaining of WNT3 and CTNNB1 in Oral Carcinoma Tissues

Immunohistochemistry was performed on 42 carcinoma tissue specimens (Fig. 3). WNT3 staining was observed in carcinoma cells in 28 cases (57%), predominantly at the invasive front. Endothelial cells, fibroblast-like cells, and macrophage-like cells were all positively stained. No staining was observed when anti-WNT3 antibody was substituted for non-immune goat IgG negative controls (data not shown). Normal oral tissues did not stain with the anti-WNT3 antibody. CTNNB1 primarily stained the cell-cell boundaries of the carcinoma cells. However, in the cells at the invasive front, CTNNB1 was localized to the nucleus and diffusively to cytoplasm. Cytoplasmic staining was evident in endothelial cells, fibroblast-like cells, and macrophage-like cells (data not shown). Normal epithelial cells stained only at the cell-cell boundaries of the suprabasal cell layer (data not shown).

DISCUSSION

Several studies emphasize that aberrant activation of the WNT signaling pathway contributes to neoplastic transformation and EMTs toward progressive tumors. The present study is the first demonstration that oral squamous cell carcinomas express WNT members and activate the signaling pathway. Profiling WNT genes by RT-PCR indicates that oral carcinoma cells express a set of WNT genes and suggests that a mixed population of WNT family members may affect the biological state of carcinoma cells. Identification of WNT members in normal gingival keratinocytes demonstrates that oral carcinoma cells most frequently expressed keratinocyte-type WNTs. In addition, carcinoma cells also miss-express sets of fibroblast-type WNTs, indicating that oral carcinoma cells of epithelial origin ectopically activate fibroblast-type WNT expression, including WNT3. Ligation of the fibroblast-type WNTs to the cell-surface receptor transmits a signal mediated through CTNNB1. This signal is one of the most characterized among the WNT-dependent signaling pathways (Seidensticker and

Behrens, 2000; Lo Muzio, 2001). Immunocytochemistry and Western blot showed that WNT-expressing carcinoma cells increased a pool of cytoplasmic-free CTNNB1 and accumulation in the nucleus, but not WNT-negative cells. Analysis of these data clearly illustrates activation of the CTNNB1-mediated WNT signaling in oral carcinoma cells.

WNT3 is a typical and powerful member of the family which activates CTNNB1-mediated signaling (Seidensticker and Behrens, 2000; Lo Muzio, 2001). Therefore, we examined the expression pattern of WNT3 and activation of the CTNNB1 signaling in tissue sections of oral carcinomas. WNT3 immunostaining demonstrated ectopic expression in carcinoma cells at the invasive front. CTNNB1 also localized in the nuclei of carcinoma cells at the invasive front, where cells evade cell-cell adhesion and gain the characteristics of EMTs (Imai *et al.*, 1995b; Brabletz *et al.*, 2001). Analogous findings have been reported in colorectal carcinomas (Kirchner and Brabletz, 2000; Brabletz *et al.*, 2001; Takahashi *et al.*, 2002). WNT signaling triggers EMTs, and nuclear localization of CTNNB1 correlates with the induction of EMTs (Eger *et al.*, 2000). EMTs are particularly prominent at the invasive front and predispose carcinomas to a more advanced state of progression (Birchmeier *et al.*, 1996). The fibroblast-type WNTs and CTNNB1-mediated signaling are documented to initiate malignant transformation and enhance cellular proliferation, dedifferentiation, and invasion and metastasis (Lo Muzio, 2001). Therefore, enhanced expression and signaling of the fibroblast-type WNT have a key role in the induction of EMTs and tumor progression.

Although we could not exclude the possibility that WNT directly initiated migration of carcinoma cells, it seems likely that WNT signaling increases local invasiveness of carcinoma cells through up-regulation of ECM-degrading endoproteases. In fact, only WNT-expressing carcinoma cells synthesized MT1-MMP. It is known that MT1-MMP is up-regulated in metastatic oral carcinomas (Shimada *et al.*, 2000). In colorectal carcinomas, MT1-MMP is a direct target gene of WNT signaling, and MT1-MMP and nuclear CTNNB1 immunolocalize in carcinoma cells in almost an identical pattern (Takahashi *et al.*, 2002). Upstream sequences during induction of MMPs and MT1-MMP *in vivo* are not clearly defined. However, these observations are highly indicative of a key role of WNT signaling in the induction of MT1-MMP, contributing to tumor invasion.

Expression of c-MYC is regulated by transcriptional and/or signal transduction hierarchies (Lutz *et al.*, 2002). Recently, van de Wetering *et al.* (2002) demonstrated that WNT signaling initiates proliferation and suppresses differentiation of colorectal carcinoma cells through induction of c-MYC, which negatively regulates the cell-cycle inhibitor p21^{CIP1/WAF1}. Although c-MYC protein accumulated in the nucleus of HOC313 and TSU cell lines, SCCKN cells, which also activated WNT signaling, did not express c-MYC. There might be WNT-independent and/or negative regulatory pathway(s) in the regulation of c-MYC expression in oral carcinoma cells.

WNT3 immunohistochemistry stained endothelial cells, fibroblast-like cells and macrophage-like cells. Activation of the WNT signaling pathway stimulates proliferation of endothelial cells (Wright *et al.*, 1999). WNT expression in endothelial cells might promote angiogenesis in oral carcinoma tissues. Immunostaining of WNT3 in fibroblast-like cells also

suggests a role in the desmoplastic response in carcinoma tissues, since it has been reported that WNT up-regulates proliferation and collagen synthesis in fibroblasts (Young *et al.*, 1998; Surendran *et al.*, 2002). Although the biological role of WNT expression in macrophages is not clear, a similar finding was reported in human colorectal carcinomas (Smith *et al.*, 1999). Therefore, the WNT family would mediate cross-talking between carcinoma cells and stromal cells. Further investigation will clarify a role for WNT-mediated cross-talking in the pathology of oral carcinomas.

The HSC2 cell line did not express any members of the WNT family. This suggests the presence of WNT-independent pathways in malignant transformation and/or the maintenance of malignant phenotypes. However, mutations of the WNT signaling molecules make constitutive activation of the signaling without expression of the WNT molecule (Morin *et al.*, 1997; Rubinfeld *et al.*, 1997; Satoh *et al.*, 2000). Although these mutations should accumulate CTNNB1 in the cytoplasm and nucleus, we could not exclude the possibility that the mutations may exceed a signaling threshold with an undetectable level of CTNNB1 protein in HSC2 cells. Future study should disclose genetic aberrations in oral carcinoma cells.

The present study demonstrated that squamous carcinoma cells of the oral cavity express a set of WNT genes and activate signaling, and suggests that the signaling is predominantly activated in carcinoma cells at the invasive front, wherein carcinoma cells accumulate EMTs. The activation state of WNT signaling correlates with MT1-MMP expression, which accelerates invasion into territorial matrix. Analysis of the data in our study suggests the possibility that inhibition of WNT signaling could be a potential target for suppression of tumor progression.

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